Improving the productivity of pharmacokinetic parameter determination

The Agilent 1200 Series Rapid Resolution LC system, 6410 triple quadrupole MS and MassHunter software provide a powerful system for bio-availability determination

Application Note

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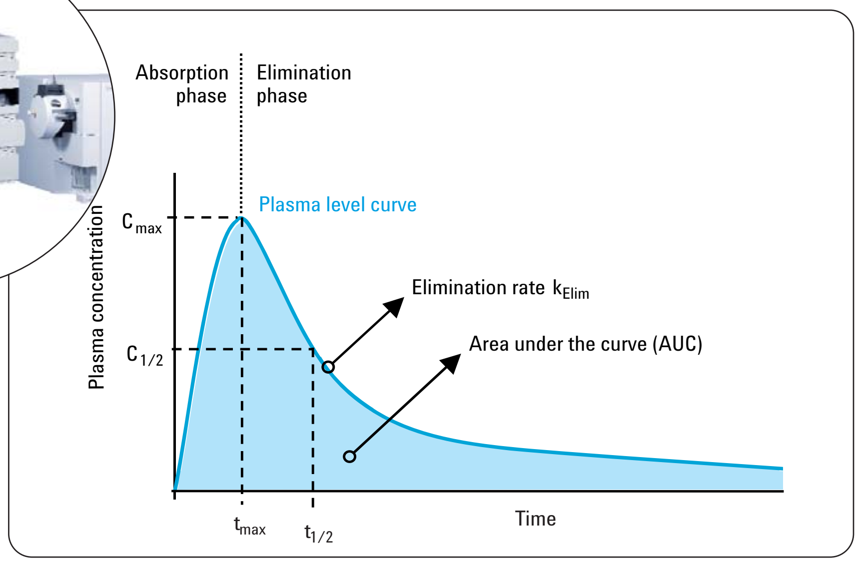


Abstract

This Application Note demonstrates how the Agilent LC/MS/MS solution can increase productivity through improved performance of the analytical equipment and superior data analysis software that reports relevant parameters directly from the analytical data. Features of the Agilent solution are:

- Fast LC methods and short cycle times through alternating column regeneration
- State-of-the-art MS performance
- Easy-to-learn and easy-to-use acquisition and data analysis software
- Calculation of PK parameters directly by the data analysis software
- Software compliant with the regulations for clinical trials

Agilent Equipment

- 1200 Series Rapid Resolution LC
- 6410 triple quadrupole MS
- CTC HTC PAL autosampler
- ZORBAX RRHT columns
- MassHunter workstation software

Application Area

- Drug development
- Clinical trials
- Bio-equivalency studies

Agilent Technologies

Introduction

When a potential new drug has shown proven activity against a certain target and passed initial ADME tests, in vivo trials with the drug are started – first with animals and then with humans. Trials with humans are known as clinical trials. During these trials researchers want to find out the bio-availability of the active compound, for example, how much of the administered amount of the compound ends up circulating freely in the blood volume and is able to reach its target organ such as the kidneys. The researchers are interested in the following parameters:

- What is the maximum concentration of the drug?
- When is the maximum concentration reached?
- What is the absorption rate?
- What is the elimination rate?
- What is the total available amount of the drug?

To determine these parameters, the drug is administered to a set of volunteers under defined conditions. At certain time intervals blood samples are taken and the amount of drug in the plasma is determined by LC/MS/MS and a plasma-concentration/time curve is constructed, (figure 1).

The absolute bio-availability of the drug can be determined by dividing the area under the curve (AUC) of a non-intravenous administration by the AUC of an intravenous administration, (equation 1). The AUC is the integral of the plasma-concentration/time curve. An example of non-intravenous administration is oral administration. The values have to be corrected for the individual doses and the relative bio-availability can be determined by comparing the AUCs of different formulations of a drug, (equation 2).

$$F_{abs} = \frac{AUC_{po}}{AUC_{iv}} \cdot \frac{dose_{iv}}{dose_{po}}$$

Equation 1
Calculation of absolute bio-availability.

$$F_{rel} = \frac{AUC_{A}}{AUC_{B}} \cdot \frac{dose_{B}}{dose_{A}}$$

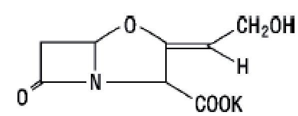
Equation 2
Calculation of relative bio-availability.

Experimental

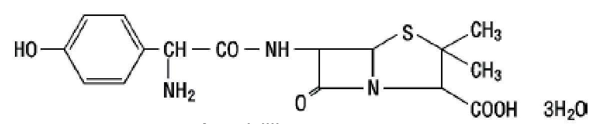
In this study the high throughput analysis of plasma samples containing amoxicillin and clavulanic acid was investigated.

A commercially available combination drug containing 250 mg of amoxicillin and 125 mg clavulabic acid is marketed under the brand name Augmentin.

Amoxicillin is a semi synthetic antibiotic with a broad spectrum of bactericidal activity against many gram-positive and gram-negative microorganisms. Amoxicillin is, however, susceptible to degradation by β-lactamases, and therefore, the spectrum of activity does not include organisms which produce these enzymes. Clavulanic acid is a β-lactam, structurally related to the penicillins, which



Potassium clavulanat



Amoxicillin

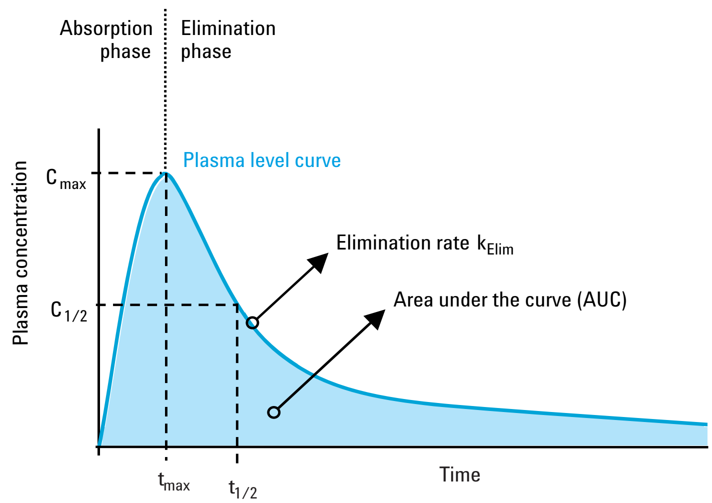


Figure 1
Schematic of a plasma concentration/time curve after single dose, non-intravenous administration of a drug, showing some typical pharmacokinetic parameters determined from that curve.

possesses the ability to inactivate a wide range of β-lactamase enzymes commonly found in microorganisms resistant to penicillins and cephalosporins. In particular, it has good activity against the clinically important plasmid-mediated β-lactamases frequently responsible for transferred drug resistance.

Equipment

- Agilent 1200 Series Rapid Resolution LC system comprising two binary pumps SL with micro vacuum degasser and thermostatted column compartment SL with 2-position/10-port valve
- CTC HTC PAL autosampler with active wash station and cooled stacks
- Agilent 6410 triple quadrupole mass spectrometer
- Agilent ZORBAX columns
- Agilent MassHunter workstation software for instrument control, data acquisition and data evaluation, operated in compliance mode.

LC method

Gradient-grade water and acetonitrile were used as mobile phase. No additional filtering of the solvents was required. MRM conditions were optimized using matrix-free samples of the individual compounds. Flow injection analysis was used with varying fragmentor voltage for precursor ion optimization and with varying collision energy for product ion optimization.

- Solvent A: Water (0.005 % HOAc)
- Solvent B: Acetonitrile (0.005 % HOAc)
- Gradient: 0.08 min, 1 % B
  0.90 min, 30 % B
  1.10 min, 30 % B
  1.11 min, 1 % B
- Regeneration: 0.01 min, 95 % B
  0.50 min, 95 % B
  0.51 min, 1 % B
- Flow: 0.8 mL/min
- Temperature: 40 °C
- Valve setting: Switch to next
- Stoptime: 1.30 min
- Posttime: off
- Injection volume: 4 µL (4x loop overfill)
- Wash procedure:
  Wash 1: 50 % water, 49 % acetonitrile, 1 % formic acid
  Wash 2: 80 % acetonitrile, 20 % tetrahydrofuran
  Wash 2x with Wash 1, wash 2x with Wash 2 after injection

MS method

- Time filter: Off
- Delta EMV: 800
- Ion source: ESI
- Drying gas temp.: 250 °C
- Drying gas flow: 11 L/min
- Nebulizer pressure: 50 psi
- Capillary voltage: 4000 V

MRM settings

- MS1Res: Unit
- MS2Res: Unit
- Dwell time: 30 ms

Plasma work-up

The plasma work-up was performed according to Yoon et al.[1], by protein precipitation with acetonitrile containing the internal standard terbutaline and back-extraction of the acetonitrile with methylene chloride. This step was necessary because a high acetonitrile concentration in the sample would have caused distorted peak shapes and made chromatographic separation of the compounds impossible.

100 µL of plasma sample were mixed with 200 µL acetonitrile containing 0.517 ng/µL terbutaline as internal standard. The mixture was vortexed for 10 s and centrifuged at 17000 rpm for 10 min. The supernatant was removed carefully with a pipette from the precipitated proteins and transferred to a new vial. 300 µL of methylene chloride was added and the mixture was vortexed for 10s and then centrifuged at 10000 rpm for 5 min. The lower layer was removed carefully and the extraction with methylene chloride was repeated. Finally, the aqueous layer was transferred to a new vial, ready for LC/MS/MS analysis.

Compound	Polarity	Precursor Ion [m/z]	Quantifier Ion [m/z]	Qualifier Ion [m/z]	Fragmentor [V]	Collision Energy [V]	Time Seg [min]
Clavulanic acid	neg	198.1	108	136	60	10	0.00-0.34
Amoxicillin	pos	366.2	114	208	120	10	0.34-1.30
Terbutaline (ISTD)	pos	226.2	152	125	120	20	0.34-1.30

Table 1
MRM settings.

Results and discussion

Method development

Alternating column regeneration was used to minimize the cycle time of the separation and to achieve high throughput. Using two binary pumps, two columns and a 2-position/10-port valve in the column compartment, column equilibration and column wash procedures could be done in parallel. While a sample was being separated on one column, the other column was washed with 95 % organic phase and then equilibrated to starting conditions. After completion of the first separation the two columns were switched. This resulted in a cycle time of less than 1.5 minutes.

Figure 2 shows a typical chromatogram of a plasma sample acquired with the method described here. Using Agilent ZORBAX RRHT StableBond C18 columns (2.1 mm x 50 mm, 1.8 µm), narrow peaks of widths in the range of 1 to 2 s were obtained. Using larger particle sizes such as 3.5 µm resulted in increased peak widths and decreased signal heights (figure 3). In contrast, columns with larger particle sizes have the advantage that less care needs to be taken during sample preparation to avoid any particles such as protein precipitation that could plug the column.

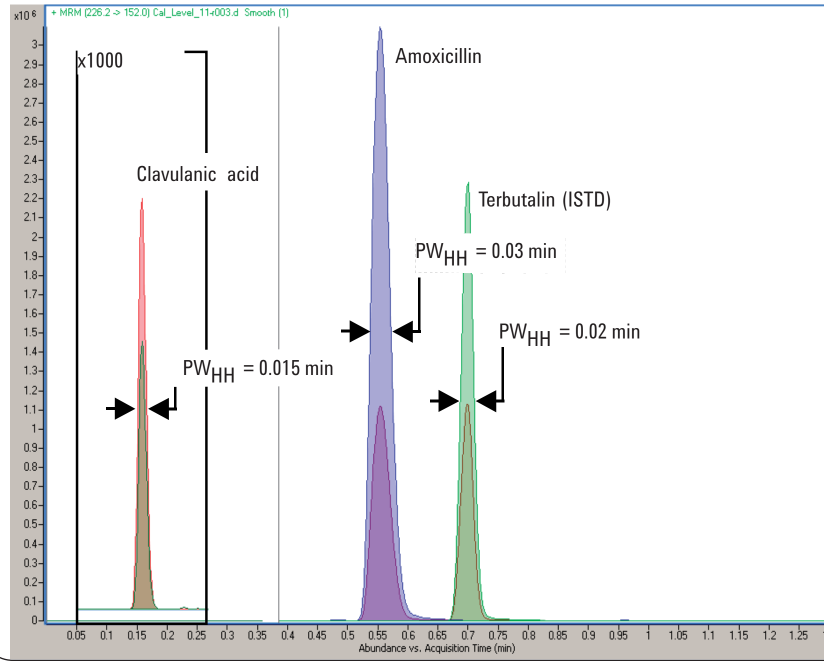


Figure 2
Chromatogram of a plasma sample with peak widths at half height for the MRM chromatograms of the quantifier ions (the underlying dark shaded peaks belong to the according qualifier ions). The total run time is 1.3 min and the cycle time was below 1.5 min.

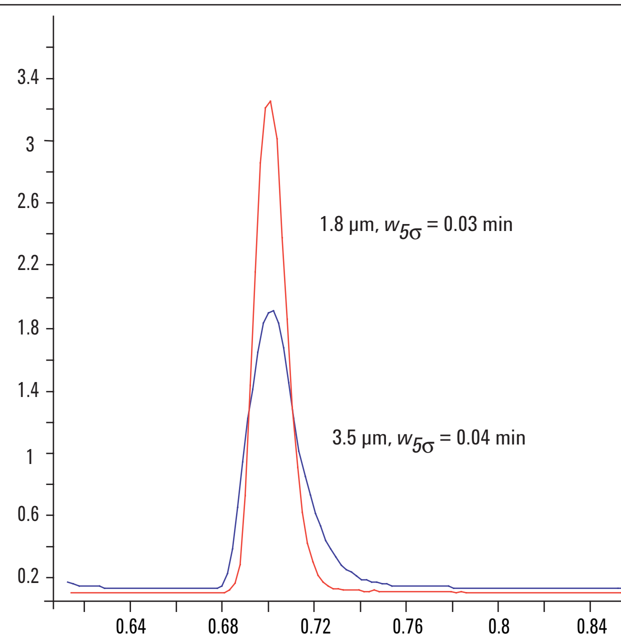


Figure 3
Comparison of 1.8 and 3.5 µm particle columns, showing the terbutaline peak.

The calibration for amoxicillin was done in the range of 10 ng/mL to 10000 ng/mL and for clavulanic acid in the range of 50 ng/mL to 5000 ng/mL. The linearity of the calibration for amoxicillin was excellent with an R^2 value of 0.99989. The R^2 value for clavulanic acid was 0.99474 (table 2 and figures 4 and 5). When adjusted to give identical injection volumes, the achieved LOQs were comparable to the analyses of amoxicillin and clavulanic acid in similar matrices as performed on non-Agilent triple quadrupole instruments[2,3].

Data Analysis

Researchers performing pharmacokinetic or bio-availability studies are interested in the pharmacokinetic values of the compounds and not in peak areas or abundances of MRM transitions. With conventional chromatographic data systems it is necessary to transfer the measured data of each sample to specialized software packages or spreadsheets in order TO calculate the desired pharmacokinetic values. In contrast, Agilent MassHunter software can speed up the data analysis process significantly. MassHunter uses Microsoft Excel in combination with Agilent built-in add-ons for reporting. Several standard pre-defined report templates are available after installation of the MassHunter software and a template has to be designed just once for each workflow. This template will then be used

Compound	Parameter	Value
Amoxicillin	LLOQ	10 ng /mL
	Range	10 – 10000 ng/mL
	Calibration formula	y = 0.2057 x
	R^2	0.99989794
Clavulanic Acid	LLOQ	50 ng /mL
	Range	50 - 5000 ng /mL
	Calibration formula	$y = 4.1936 \times 10^{-5}$ x $- 2.7931 \times 10^{-7}$
	R^2	0.99474559

Table 2
Calibration results for amoxicillin and clavulanic acid.

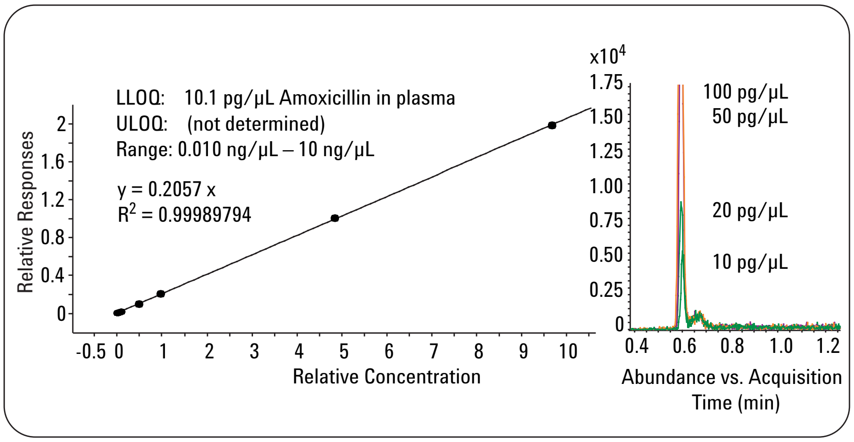


Figure 4
Calibration curve for amoxicillin with chromatograms of the lower concentrations.

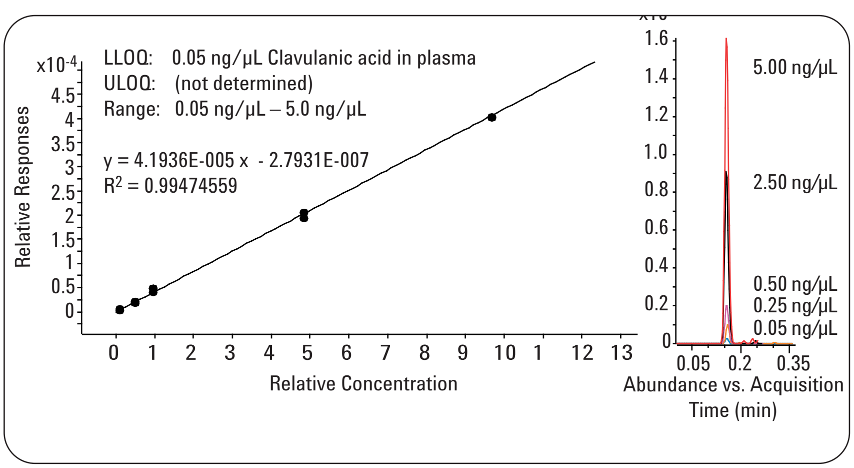


Figure 5
Calibration curve for clavulanic acid with chromatograms.

for each individual report. Figure 6 shows the data flow during analysis and reporting.

Designing a report template is straightforward –all available information from the data files and batches are selected from a so-called XML map and dragged to the worksheet. The possibilities are numerous, for example, some general batch information can appear at the top of the report and then repeating sections can be defined where the sample and compound information will be placed and indexed during report processing. Filters can be applied, for example, to show all samples but no blanks. Graphics can be inserted and printouts can be attractively formatted. When starting with a pre-defined template only minimal experience is required.

For the work presented in the study an advanced report template was designed using the full capability of Excel to generate reports that deliver the final result of the assay by applying calculations and Excel formula to the acquired raw data of the samples. This was only possible because of the unique combination of MassHunter as the quantification software and Excel as the spreadsheet program to generate analytical reports.

During processing the data from the batch results file are imported, filtered and then used for the custom calculations.

The Excel template used for this study made use of additional Excel add-on software to calculate typical pharmacokinetic parameters from time-dependent concentration data of patient samples[4]. These calcula-

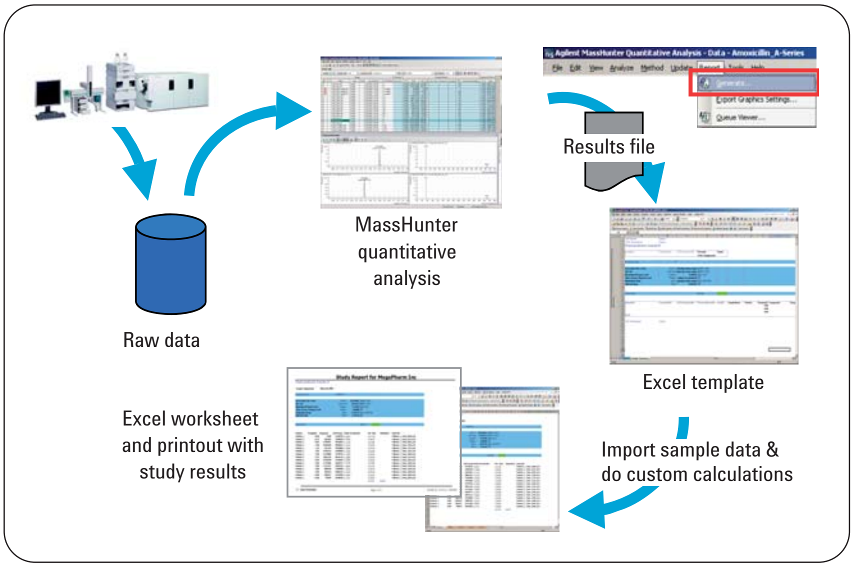


Figure 6
Data flow in MassHunter during data analyses. The acquired raw data are assigned to a batch and analyzed using a quantifying method (automation is possible for defined workflows). The generated results file contains all data. As soon as report generation is started by the user, a pre-defined Excel template file is used to filter and arrange all the data of the batch file, customer defined calculations are performed and final reports as Excel worksheets or as printouts are generated (printing to a PDF file is also possible). The process is started by selecting the Generate Report menu item (top right) and runs fully automatically.

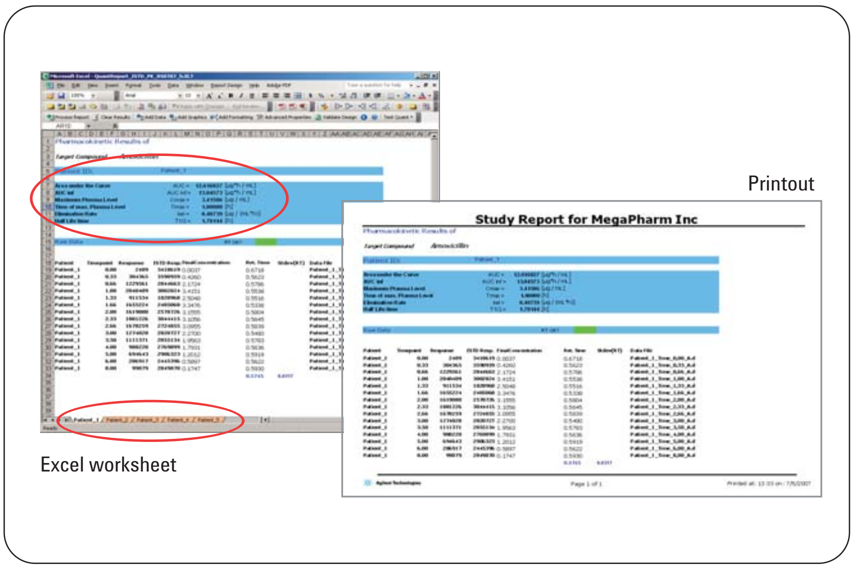


Figure 7
The results of the study are summarized after reporting automatically in an Excel workbook containing several worksheets, one for each patient (left). Each worksheet shows the raw data and the calculated pharmacokinetic parameters of that patient. The contents of the worksheets can be printed out (right).

tions could have also been done using standard Excel functionalities. The final report shows the values for AUC_{0-8h}, $AUC_{0-\infty}$, C_{max}, t_{max}, k_{El} and the half life $t_{1/2}$. Each dataset, containing the raw data as well as the calculated pharmacokinetic parameters for a patient, was summarized on one Excel worksheet. The Excel workbook contained all patient data of the complete study on separate worksheets (figure 7). The design can be configured during setup of the Excel template.

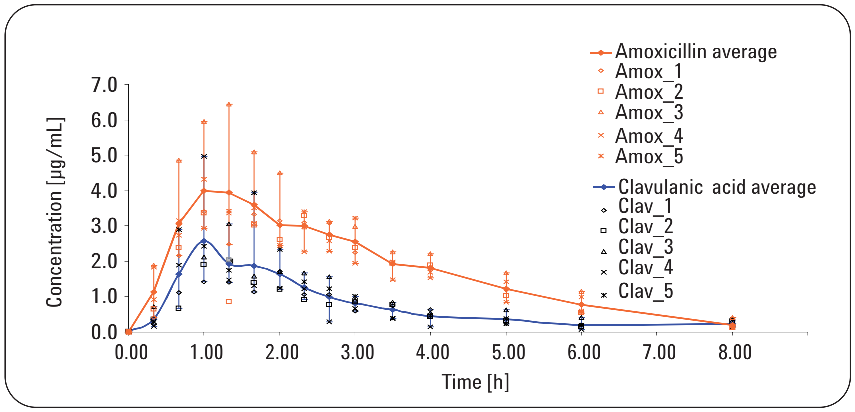


Figure 6
Plasma concentration curves for amoxicillin and clavulanic acid, n=5.

Summarized study results

Figure 8 and table 2 show the summarized pharmacokinetic results of all samples investigated in this study and give the averaged pharmacokinetic values for amoxicillin and clavulanic acid. The results are in good agreement with published results[1].

Compound	Parameter	Value
Amoxicillin	AUC_{0-8}	14.17 ± 3.30 µg*h/mL
	C_{max}	4.00 ±1.33 µg/mL
	t_{max}	1.00 ±0.18 h
	k_{El}	0.41 ±0.03 1/h
	$AUC_{0-\infty}$	14.66 ±4.24 µg*h/mL
Clavulanic acid	AUC_{0-8}	5.98 ±1.17 µg*h/mL
	C_{max}	2.57 ±1.35 µg/mL
	t_{max}	1.00 ±0.38 h
	k_{El}	0.39 ±0.06 1/h
	$AUC_{0-\infty}$	6.60 ±1.27 µg*h/mL

Table 3
Pharmacokinetic parameters for amoxicillin and clavulanic acid.

Conclusion

Performing pharmacokinetic studies generates a multitude of data. Many animals or patients have to be dosed with the potential drug and typically in the range of 15-20 individual samples are taken at increasing time points. This might also be multiplied by repeated analyses of individual samples to improve statistics. Using alternating column regeneration with the Agilent 1200 Series Rapid Resolution LC and Agilent 6410 triple quadrupole LC/MS, short cycle times of 1.5 min from injection-to-injection per sample were achieved in this study. Further, a significant improvement in productivity was gained using the Agilent MassHunter Workstation software for data acquisition, qualitative analysis and quantitative analysis. This software performed all the required calculations during reporting to directly output pharmacokinetic parameters such as AUC, C_{max}, T_{max}, k_{El}, $t_{1/2}$, and so on, in contrast to established chromatographic data systems that just report the areas or concentrations of the individual samples. No additional manual or macro-driven data exchange with other software was necessary. The sensitivity achieved with this method and the reported pharmacokinetic parameters were in good agreement with published results. The software is designed to comply with the regulations as required for most pharmacokinetic studies.

References

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4.
"PK Functions for Microsoft Excel" developed by Joel I. Usansky, Atul Desai and Diane Tang-Liu, *Department of Pharmacokinetics and Drug Metabolism Allergan, Irvine, CA 92606, USA (not for commercial use)*

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